

Transcriptome Profiling of the Green Alga *Spirogyra pratensis* (Charophyta) Suggests an Ancestral Role for Ethylene in Cell Wall Metabolism, Photosynthesis, and Abiotic Stress Responses^{1[OPEN]}

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It is well known that ethylene regulates a diverse set of developmental and stress-related processes in angiosperms, yet its roles in early-diverging embryophytes and algae are poorly understood. Recently, it was shown that ethylene functions as a hormone in the charophyte green alga *Spirogyra pratensis*. Since land plants evolved from charophytes, this implies conservation of ethylene as a hormone in green plants for at least 450 million years. However, the physiological role of ethylene in charophyte algae has remained unknown. To gain insight into ethylene responses in *Spirogyra*, we used mRNA sequencing to measure changes in gene expression over time in *Spirogyra* filaments in response to an ethylene treatment. Our analyses show that at the transcriptional level, ethylene predominantly regulates three processes in *Spirogyra*: (1) modification of the cell wall matrix by expansins and xyloglucan endotransglucosylases/hydrolases, (2) down-regulation of chlorophyll biosynthesis and photosynthesis, and (3) activation of abiotic stress responses. We confirmed that the photosynthetic capacity and chlorophyll content were reduced by an ethylene treatment and that several abiotic stress conditions could stimulate cell elongation in an ethylene-dependent manner. We also found that the *Spirogyra* transcriptome harbors only 10 ethylene-responsive transcription factor (*ERF*) homologs, several of which are regulated by ethylene. These results provide an initial understanding of the hormonal responses induced by ethylene in *Spirogyra* and help to reconstruct the role of ethylene in ancestral charophytes prior to the origin of land plants.

The hormone ethylene plays a vital role throughout the life cycle of plants. Ethylene is a small gaseous molecule participating in a wide range of physiological and developmental processes, including seed germination, cell elongation, cell division, cell death, fruit ripening, senescence, and responses to biotic and abiotic stresses (McManus, 2012; Wen, 2015). Ethylene biology has been well studied in the angiosperms *Arabidopsis* (*Arabidopsis thaliana*), rice (*Oryza sativa*), and tomato (*Solanum lycopersicum*) and is known to play important roles in other land plant lineages: gymnosperms, Monilophyta (ferns and related species), Lycopodiophyta (lycophods and related species), Marchantiophyta (liverworts), Bryophyta (mosses), and Anthocerotophyta (hornworts; Johri, 2008; Groen and Whiteman, 2014; Van de Poel et al., 2015). By contrast, the role of ethylene in non-land plants (algae) is either unknown or poorly characterized (Van de Poel et al., 2015; Lu and Xu, 2015). However, recently Ju et al. (2015) obtained bioinformatic, molecular, and physiological evidence that ethylene is a functional hormone in the charophyte alga *Spirogyra pratensis*, indicating that the evolutionary origin of the plant hormone ethylene predates the origin of land plants.

Land plants evolved from an ancestral aquatic charophyte (Leliaert et al., 2012; Wickett et al., 2014) more than 450 million years ago (Bremer, 1985; Karol et al., 2001; Sanderson et al., 2004). The genus *Spirogyra* belongs to the Zygnematophyceae and is comprised of more than 500 filamentous species with a global distribution (Stancheva et al., 2013; Guiry and Guiry, 2015). Recent phylogenetic analyses placed the Zygnematophyceae as the closest living relatives of land plants (Delwiche and Cooper, 2015, and refs. therein) although still not fully clarified (Cooper, 2014). This makes *S. pratensis* and other species of the Zygnematophyceae useful organisms to study the origins of important land plant traits. Ju et al. (2015) showed that *Spirogyra* is able to produce ethylene using the specific precursor 1-aminocyclopropane-1-carboxylic acid (ACC), possesses an ethylene signaling pathway homologous to that of embryophytes, and displays ethylene-induced cell elongation. The genome (Hori et al., 2014) and transcriptome (Ju et al., 2015) of *Klebsormidium flaccidum*, a member of an earlier diverging lineage of charophyte algae (Klebsormidiophyceae), also encodes putative ethylene signaling homologs, suggesting a possible earlier origin of the ethylene signaling pathway. However, functional evidence is not available

for ethylene production and signaling in *Klebsormidium*. An even earlier origin of ethylene signaling is possible with a cyanobacterial origin hypothesized for the ethylene receptor (Mount and Chang, 2002). The transition from an aquatic to a terrestrial environment required the acquisition of novel processes to cope with diverse stress conditions (Yue et al., 2012). Thus, the acquisition of the ethylene system by an ancient charophyte lineage might have been one of the crucial developments contributing to the successful colonization of land (Timme and Delwiche, 2010; Hori et al., 2014; Ju et al., 2015). In land plants, ethylene is known to play a major role in adaptive responses to abiotic stresses, including drought, flooding, salinity, and heat (Kazan, 2015), raising the question of whether ethylene also played a role as a stress hormone in the common ancestor of land plants and charophyte algae.

Although ethylene is typically considered to be an inhibitor of cell elongation, there are examples in which ethylene stimulates cell elongation. Ethylene stimulates cell elongation in filaments of the moss *Physcomitrella patens* (Yasumura et al., 2012, 2015), in pillar cell elongation in the aquatic liverwort *Riella helicophylla* (Stange and Osborne, 1988), in rachis elongation of the semi-aquatic fern *Regnellidium diphyllum* (Musgrave and Walters, 1974; Cookson and Osborne, 1979), and in coleoptile and internode elongation of monocots such as deep water rice (Ku et al., 1970; Métraux and Kende, 1983; Van Der Straeten et al., 2001), oat (Suge, 1971), and wheat (Suge et al., 1997). In *Arabidopsis* seedlings, ethylene stimulates cell elongation in the hypocotyl when grown in the light (Smalle et al., 1997), in

contrast to the well-known ethylene-induced inhibition of cell elongation in dark-grown *Arabidopsis* seedlings (Guzmán and Ecker, 1990). It is clear from these examples that the ethylene-induced cell elongation response is a feature that the charophyte alga *Spirogyra* shares with different members of land plant lineages. Cell elongation was possibly one of the original ethylene responses that were acquired during the evolution of charophytes and conserved in land plants. To investigate the ethylene response at the transcriptomic level in *Spirogyra*, we carried out mRNA sequencing over a time course of ethylene treatment. Furthermore, to test the hypothesis that ethylene might have evolved as a stress hormone in the common ancestor of charophyte algae and land plants, we examined whether the ethylene-induced cell elongation response can be stimulated under a variety of stress conditions.

RESULTS

Time-Course mRNA Sequencing of *Spirogyra* Cultures Treated with and without Ethylene

To uncover the biological processes underlying the ethylene response in *Spirogyra*, we performed whole transcriptome shotgun sequencing over a 24-h time-course of ethylene treatment. We chose to sample at 3, 6, 12, and 24 h posttreatment in order to capture most of the transcriptional changes, based on published transcriptome profiling data of dark-grown ethylene-treated *Arabidopsis* seedlings (Chang et al., 2013). The 24 samples produced about 502.3 million reads, averaging 20.9 million reads per sample (Supplemental Table S2). Filtered and trimmed reads are available via the NCBI Short Read Archive. The assembled transcriptome comprised 37,050 isoforms (transcripts) from 26,839 genes, of which 47.7% were annotated with an *Arabidopsis* homolog and/or a pfam domain (Supplemental Table S3).

To verify that *Spirogyra* cells were responding to the ethylene treatment, we analyzed the average cell length after 24-h ethylene treatment (Fig. 1). The average cell length increase of 15% after 24 h of ethylene treatment was subtle but significant and was consistent with what would be expected for a 24-h treatment based on previously reported experiments of longer duration (Ju et al., 2015).

Identification of Ethylene-Regulated Genes in *Spirogyra*

To identify ethylene-regulated genes, we performed a differential gene expression analysis comparing the ethylene-treated versus the untreated samples at each time point. The differential expression pattern for all 37,050 transcripts is shown in Supplemental Figure S2. The correlation matrix shows that at each time point the three replicates for each treatment cluster together, except at 3 h posttreatment, when all samples are highly

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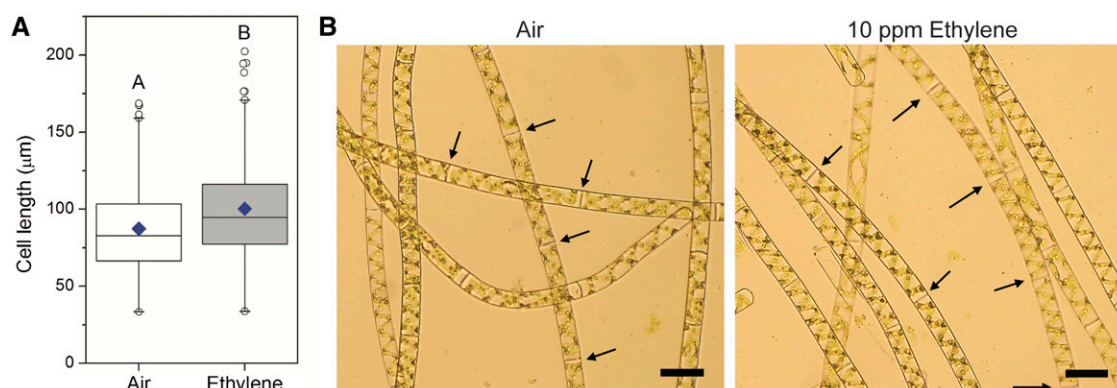


Figure 1. The effect of 24-h ethylene exposure (10 ppm) on the average cell length (μm) of *S. pratensis*. A, Cell length distribution was obtained by measuring 200 to 300 individual cells for each treatment. All boxes bound the 25th and 75th percentiles, whiskers show the outermost data points <1.5 times the interquartile range beyond the box. Blue diamonds indicate the mean; horizontal lines indicate the median; white circles indicate outliers. Significant differences ($P < 0.05$) are indicated by letters above the bars. B, Representative microscopic image of *Spirogyra* filaments showing a subtle cell length increase after a 24-h ethylene treatment. Arrows indicate the ends of individual cells in the filaments. Scale bar is 50 μm.

correlated (Supplemental Fig. S3). The correlation between the two treatments weakens over time, indicating that there is an increase in the number of genes that are differentially regulated by ethylene over time. This trend is evident in the volcano plots (Fig. 2), which show an increase in differentially regulated genes over time. The number of transcripts that are up-regulated by ethylene is higher than the number of transcripts that are down-regulated by ethylene at each time point (Fig. 3). Supplemental File S1 lists the differentially expressed transcripts with their logFC (fold change), false discovery rate (FDR), and putative Arabidopsis

homolog (best blast hit with e-value $< 1 \times 10^{-10}$) for each time point.

To quantify background gene expression changes in the *Spirogyra* transcriptome, we analyzed differential gene expression in the untreated samples over time by comparing the 3 vs 6 h, 6 vs 12 h, and 12 vs 24 h control samples (Supplemental Fig. S4). There are a substantial number of transcripts whose expression changed over the time course of the experiment. Differential expression over time is not unexpected and most likely results from a combination of response to the experimental setup (i.e. the transfer from liquid culture to semidry

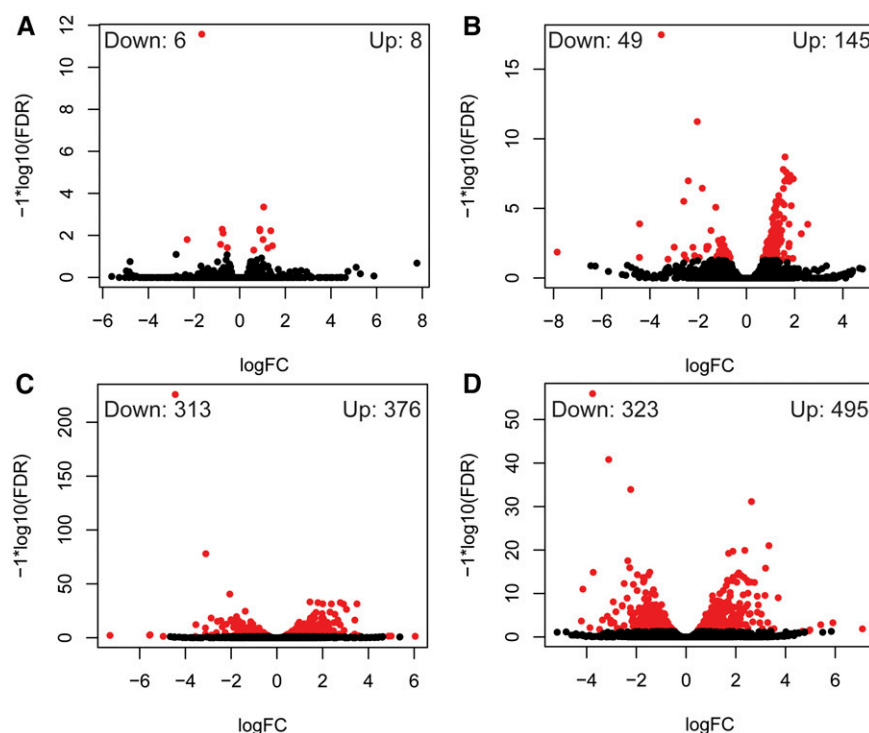


Figure 2. Volcano plots showing the relation between the FDR and the FC for all transcripts. The transcripts that are differentially expressed (the P value of the FDR < 0.05 and the FC > 4) are indicated in red for the different time points: A, 3-h control versus 3-h ethylene; B, 6-h control versus 6-h ethylene; C, 12-h control versus 12-h ethylene; and D, 24-h control versus 24-h ethylene. The differentially expressed transcripts for each time point are listed in Supplemental File S1.

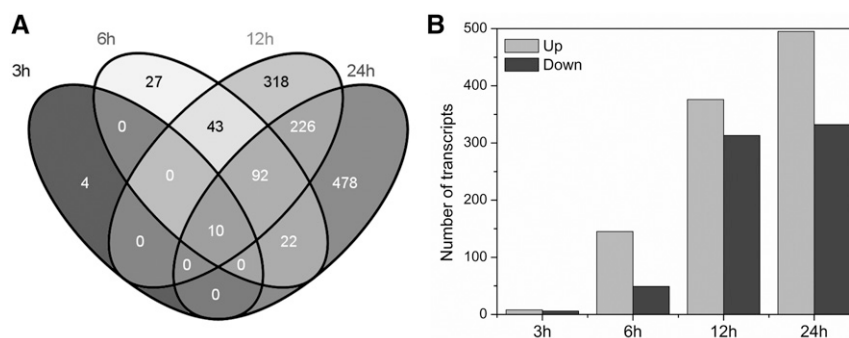


Figure 3. Overview of the differential expression analysis statistics (with a FDR value of $P < 0.05$ and an expression level change of at least 4-fold). A, Venn diagram showing the number of differentially expressed genes that are regulated by ethylene over time (by comparing the ethylene versus control samples for each time point). The Venn diagram was created online using Venny (<http://bioinfogp.cnb.csic.es/tools/venny/>). B, Number of transcripts that are differentially regulated by ethylene for each time point (ethylene-treated compared to untreated samples for each time point). Light gray bars are up-regulated genes and dark gray bars are down-regulated genes.

conditions) and normal circadian expression cycles. By incorporating control samples (untreated) at each time point, we were able to identify ethylene-regulated genes against this background of fluctuating gene expression. However, it is likely that the expression level of some genes reflects both the effect of ethylene treatment and changes in background expression.

We used qPCR to evaluate the RNA-seq expression results for four different transcripts, using cultures independently treated with or without ethylene. Based on the differentially expressed transcripts of the RNA-seq experiment, four genes were chosen that showed distinct expression patterns (up-regulated vs down-regulated) and that are likely to be involved in different ethylene-regulated processes: *CYTOKININ RESPONSE FACTOR1* (*CRF1*; ethylene-responsive transcription factor [ERF] family), *GLC-1-PHOSPHATE ADENYL TRANSFERASE* (sugar metabolism), *CALMODULIN-DOMAIN PROTEIN KINASE7* (abiotic stress signaling), and *EXPANSIN A18* (*EXPA18*; cell wall metabolism). As shown in Supplemental Figure S5, the qPCR expression profiles of these genes are similar to the RNA-seq results, generally validating the RNA-seq data.

Immediate Ethylene-Regulated Genes in *Spirogyra*

To identify immediate ethylene-regulated transcripts in *Spirogyra*, we compared the ethylene-treated and untreated samples at the 3-h time point and found 14 transcripts whose expression differed significantly, eight of which had a high-scoring blast hit to the Arabidopsis proteome (e-value $< 1 \times 10^{-10}$; see Supplemental File S1 for the list). To our knowledge, the putative Arabidopsis homologs of these eight genes have not previously been linked to ethylene. One transcript encodes a protein kinase homolog (comp14026_c0; Fig. 4A) that is largely uninvestigated in Arabidopsis. A second ethylene-regulated transcript encodes a homolog of *TRANSLOCON AT THE INNER MEMBRANE OF*

CHLOROPLAST 214 (*TIC214*), which is a part of the chloroplast translocon involved in protein transport across the chloroplast membrane and a crucial element in facilitating horizontal gene transfer in plants (de Vries et al., 2015). *TIC214* is unique to the green lineage, and the current embryophyte-like *TIC214* evolved from an ancestral charophyte *TIC214* (de Vries et al., 2015). This plastid-encoded gene is rapidly and consistently down-regulated in response to ethylene in *Spirogyra* (Fig. 4B). An ethylene response has not been observed for the Arabidopsis homolog (according to the Arabidopsis eFP browser; Winter et al., 2007). A third early ethylene-regulated and negatively regulated transcript is a homolog of *TRANSPARENT TESTA8* (*TT8*; Fig. 4C), which encodes a protein that makes up a complex that regulates flavonoid biosynthesis (namely proanthocyanidin and anthocyanin) and is not regulated by ethylene in Arabidopsis seedlings (Chang et al., 2013). Three other transcripts are homologs of *RESPONSIVE TO DEHYDRATION 21B* (*RD21B*), *LATE EMBRYOGENESIS ABUNDANT* related protein (*LEA*), and *20S PROTEASOME ALPHA SUBUNIT C1* (*PAC1*), which show a rapid ethylene-induced up-regulation in *Spirogyra*, peaking at 6 h posttreatment (Fig. 4, D–F). In Arabidopsis, *RD21B* is involved in peptidase activity during biotic and abiotic stress (Zhang et al., 2014) but does not show any ethylene responsiveness in seedlings (Winter et al., 2007; Chang et al., 2013). *LEA* proteins play a role during dehydration tolerance and other stress responses (Hundertmark and Hincha, 2008). The *PAC1* homolog (20S proteasome alpha subunit C1) might be involved in the organization of a general proteolytic complex. A seventh gene is a *WD40* homolog (a transducing/*WD40* repeat-like superfamily protein) that seems to be repressed by ethylene at the early 3-h time point (Fig. 4G). *WD40* proteins can play a role in wide variety of cellular processes (van Nocker and Ludwig, 2003). The eighth early ethylene-regulated transcript encodes a homolog of an unknown protein (At3g19430). Its expression is drastically inhibited by ethylene in *Spirogyra* (Fig. 4H), as

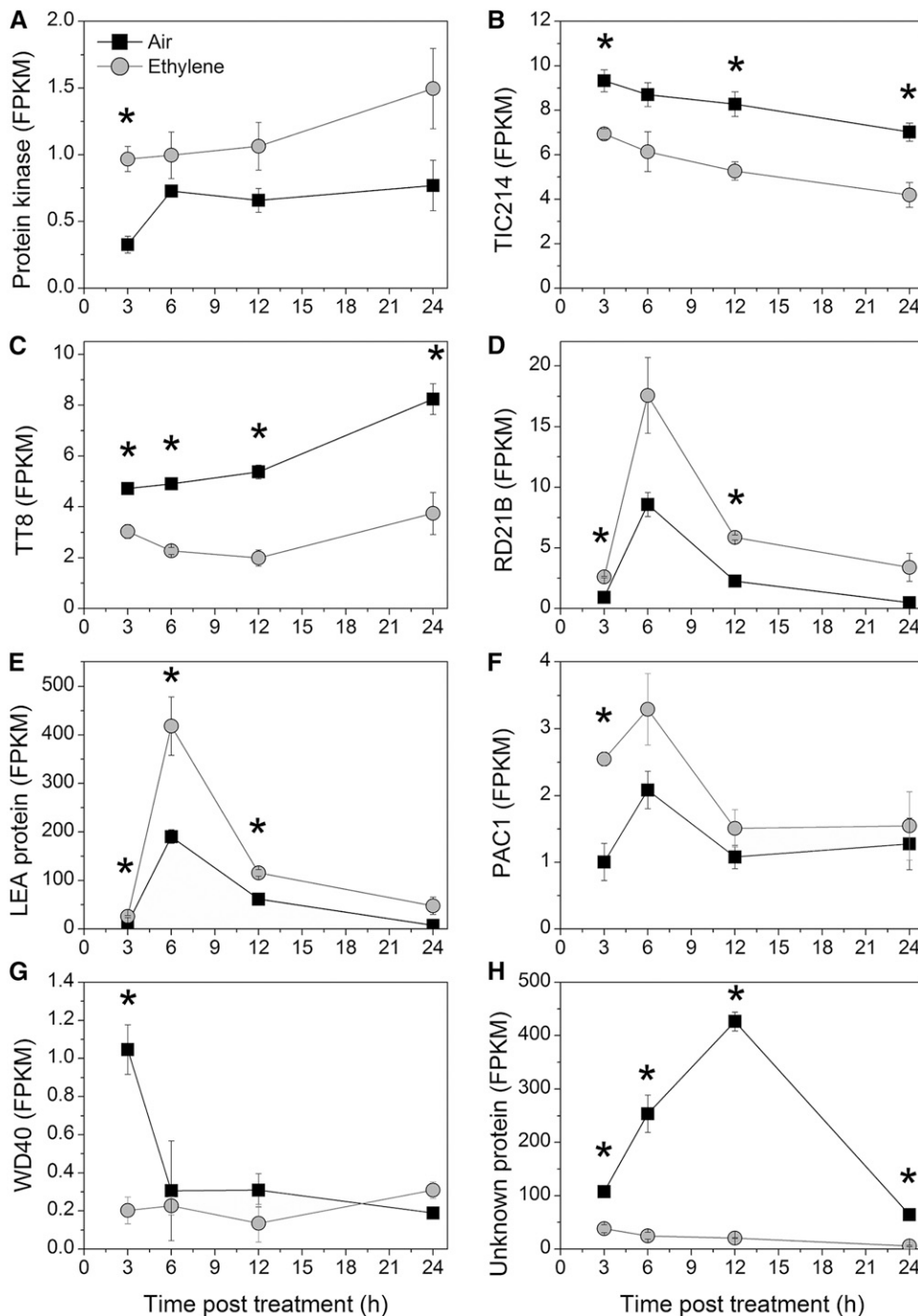


Figure 4. Expression profiles of *S. pratensis* genes that show significant expression differences after 3 h of treatment. Relative normalized gene expression (TMM-*FPKM*) profiles are shown for a *PROTEIN KINASE SUPERFAMILY* homolog (At3g19300; comp14026_c4) (A), the *CHLOROPLAST MEMBRANE TRANSLOCATOR TIC214* (or *YCF1*) homolog (AtCg01130; comp11735_c0) (B), a basic helix-loop-helix DNA binding superfamily protein homolog of *TT8* (At4g09820; comp6246_c0) (C), granulin repeat Cys protease homolog (*RESPONSIVE TO DEHYDRATION21B* [*RD21B*]; At5g43060; comp38750_c0) (D), *LATE EMBRYOGENESIS ABUNDANT* (*LEA*) related homolog (At3g19430; comp14490_c0) (E), *20S PROTEASOME ALPHA SUBUNIT C1* homolog (*PAC1*; At3g22110; comp54358_c0) (F), *TRANSDUCING/WD40 REPEAT PROTEIN* homolog (*WD40*; At5g15550; comp13964_c1) (G), and protein of unknown function homolog (At1g47980; comp11757_c0) (H). Values represent the average of three biological replicates with *SD* of the mean. Significant differences between treatments ($P < 0.05$) are indicated with an asterisk.

was observed to a lesser extent for the homolog in *Arabidopsis* (Chang et al., 2013). This unknown protein contains a conserved ferritin domain, which is thought to be involved in iron homeostasis (Ravet et al., 2009).

Spirogyra Expresses a Few AP2/ERF Related Transcription Factors in Response to Ethylene

In land plants, ethylene signaling induces expression of a subset of *ERF* transcription factor genes (members

of the APETALA2 (AP2)/ERF superfamily) via the master transcription factor ETHYLENE INSENSITIVE3 (EIN3; Riechmann and Meyerowitz, 1998; Solano et al., 1998). In *Arabidopsis* there are 147 AP2/ERF members divided in three families (the ERF family, the AP2 family, and the RAV family; Nakano et al., 2006). To identify the *Spirogyra* *ERF* homologs, the conserved AP2/ERF domain was used as a query in a blast search against the *Spirogyra* transcriptome obtained in this study. Only 10 different putative *ERFs* were retrieved (Supplemental Fig. S6), and an alignment with *Arabidopsis* homologs

showed that the AP2/ERF domain is well conserved in these proteins (Supplemental Fig. S7). Of these 10 *ERFs*, only the *RELATED TO AP2.4*, *CRF1* and *DEHYDRATION RESPONSE ELEMENT-BINDING PROTEIN A6* homologs were differentially regulated by ethylene in our experiment (Supplemental File S1). The sole *ERF* that showed a strong and steady up-regulation by the ethylene treatment is annotated as a *CRF1* homolog (Supplemental Fig. S6F) based on the best reciprocal blast hit.

Gene Ontology Enrichment Analysis Suggests Ethylene-Regulated Pathways in *Spirogyra*

Of the 828 differentially regulated transcripts at the 24-h time point, 74.15% were annotated based upon sequence similarity to proteins present in the Arabidopsis proteome (e-value $< 1 \times 10^{-10}$). A full list of the annotated *Spirogyra* homologs for each time point is given in Supplemental File S1 and their corresponding expression values (TMM-FPKM) are given in Supplemental File S2. The differentially expressed transcripts of the 24-h time point were divided in two subsets, one containing genes that were up-regulated by ethylene at the 24-h time point (Fig. 5A) and the other containing the down-regulated genes of the 24-h time point (Fig. 5B), and both subsets were subjected to a Gene Ontology (GO) enrichment analysis and semantic clustering. Among the significantly enriched GO terms found for the set of up-regulated genes, terms related to primary cell wall metabolism and sugar metabolism were quite

abundant. For the set of down-regulated genes, the significantly enriched GO terms that were abundant are related to the chlorophyll biosynthesis and photosynthesis pathways. It was also striking that many of the significantly enriched GO terms, for both the set of up- and down-regulated genes, are related to responses to abiotic stress.

The pathways identified via the GO enrichment analysis and semantic clustering for the 24-h time point were also already enriched at the 6- and 12-h time points (Supplemental Fig. S8). Interestingly, after 6 h, the enriched GO terms for the down-regulated transcripts were solely related to the regulation of transcription. For the 3-h time point, there were no significantly enriched GO terms. The majority of biological processes identified via the GO enrichment analysis were also retrieved by a MapMan analysis (Supplemental Fig. S9).

Ethylene Treatment Regulates Cell Wall Flexibility in *Spirogyra*

The GO enrichment analyses showed an enrichment of cell wall-related GO terms (at the 12- and 24-h time points). Among the differentially expressed genes from the 12- and 24-h time points (Supplemental File S1), we found several transcripts related to cell wall modification, in particular *EXPANSINS* (*EXPs*) and *XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASES* (*XTHs*), and a few *CELLULOSE SYNTHASES*. Figure 6 shows the expression profiles of some interesting cell wall-modifying

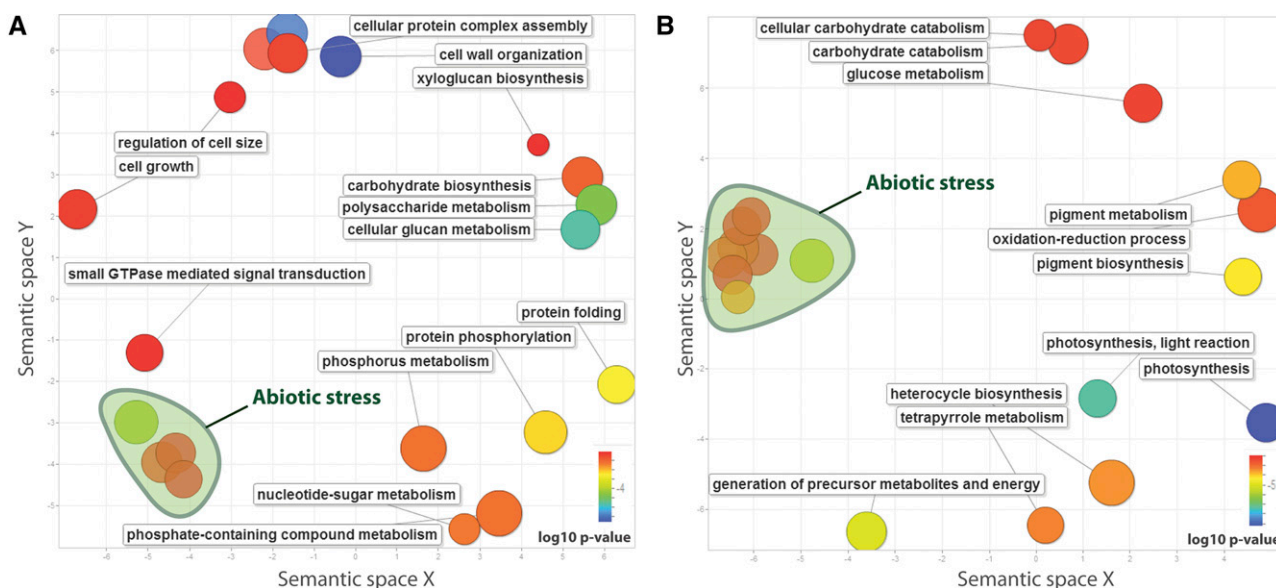


Figure 5. GO enrichment analysis of biological processes regulated by ethylene (24 h untreated versus 24 h ethylene treated) on a subset of genes that are up-regulated (A) and a subset of genes that are down-regulated (B) by ethylene. To identify similar GO terms among the enriched terms, this set of GO terms was categorized using semantic clustering (REVIGO). Each ball represents a cluster of GO terms related to a similar process, and the size of the ball represents the number of GO terms grouped in that cluster. The color of the balls indicates the *P* value of the GO enrichment analysis; red indicates the highest *P* value and blue the lowest (least likely to occur by chance). The cutoff *P* value for the GO enrichment analysis was set to 0.01. The background used for the GO enrichment analysis was all the annotated *Spirogyra* transcripts of the assembly. Clusters of GO terms related to abiotic stress are indicated by the green plane.

enzymes. The majority of these genes are up-regulated by ethylene (sometimes only temporarily at 6 h), but some are also down-regulated by ethylene.

Ethylene Treatment Down-Regulates Chlorophyll Biosynthesis and Photosynthesis in *Spirogyra*

Many of the enriched GO terms for the ethylene treatment were related to chlorophyll biosynthesis and photosynthesis. The expression profiles of all the differentially expressed genes related to chlorophyll biosynthesis or photosynthesis are shown in Supplemental Figure S10. Many of these homologs show a down-regulated expression profile for the untreated samples, but ethylene treatment further stimulates this declining trend. The role of ethylene in these two processes was confirmed by measurements of chlorophyll content and

photosynthesis (Fig. 7). The total chlorophyll content is lower in ethylene-treated *Spirogyra* samples compared to untreated samples. Treating *Spirogyra* with 1-methylcyclopropene (1-MCP) seems to prevent the down-regulation of chlorophyll biosynthesis resulting in a higher chlorophyll content. In addition, the net photosynthetic capacity (measured by the maximal CO₂ exchange rate at high illumination) significantly decreased in *Spirogyra* samples that had been treated with ethylene for 10 d. Treatment with 1-MCP appears to revert the ethylene-induced reduction in photosynthesis.

Ethylene Signals Abiotic Stress in *Spirogyra*

Both the MapMan and GO enrichment analyses showed that many abiotic stress-responsive genes were differentially expressed by the ethylene treatment

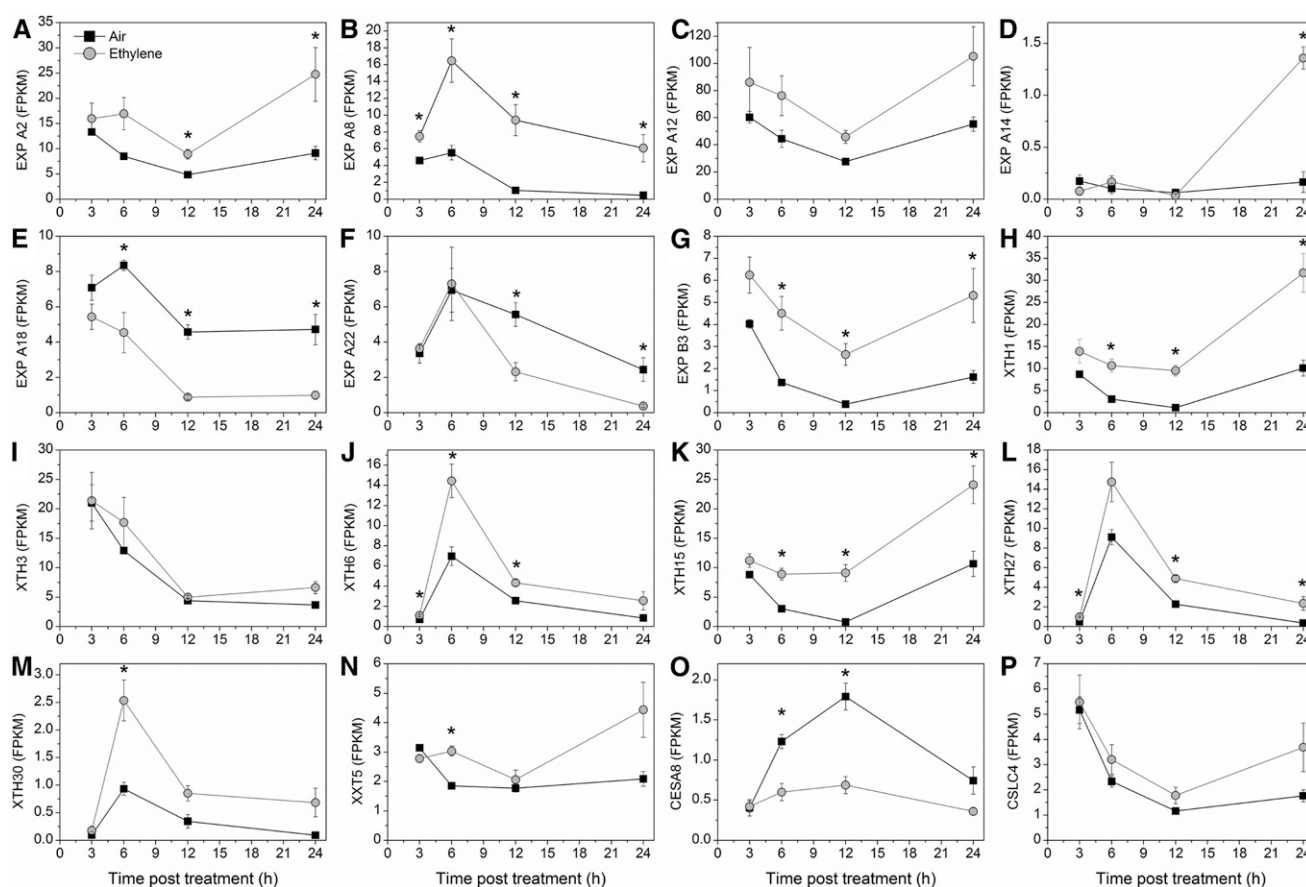


Figure 6. Ethylene-regulated cell wall modifying enzymes in *Spirogyra*. Relative normalized gene expression (TMM-FPKM) profiles are shown for EXPANSIN A2 homolog (EXPA2; At5g39270; comp46631_c0; A), EXPANSIN A8 homolog (EXPA8; At2g40610; comp37004_c0; B), EXPANSIN A12 homolog (EXPA12; At3g15370; comp14442_c0; C), EXPANSIN A14 homolog (EXPA14; At5g56320; comp13155_c0; D), EXPANSIN A18 homolog (EXPA18; At1g62980; comp42456_c0; E), EXPANSIN A22 homolog (EXPA22; At5g39270; comp46631_c0; F), EXPANSIN B3 homolog (EXPB3; At4g28250; comp10991_c1; G), XTH1 (At4g13080; comp26605_c0; H), XTH3 (At3g25050; comp14616_c0; I), XTH6 (At5g65730; comp46154_c0; J), XTH15 (At4g14130; comp6908_c0; K), XTH27 (At2g01850; comp6450_c0; L), XTH30 (At1g32170; comp6861_c0; M), XYLOGLUCAN XYLOSYLTRANSFERASE5 homolog (XXT5; At1g74380; comp6335_c0; N), CELLULOSE SYNTHASE8 homolog (CESA8; At4g18780; comp14512_c9; O), and CELLULOSE SYNTHASE-LIKE C4 homolog (CSLC4; At3g28180; comp51653_c0; P). Values represent the average of three biological replicates with SD of the mean. Significant differences between treatments ($P < 0.05$) are indicated with an asterisk.

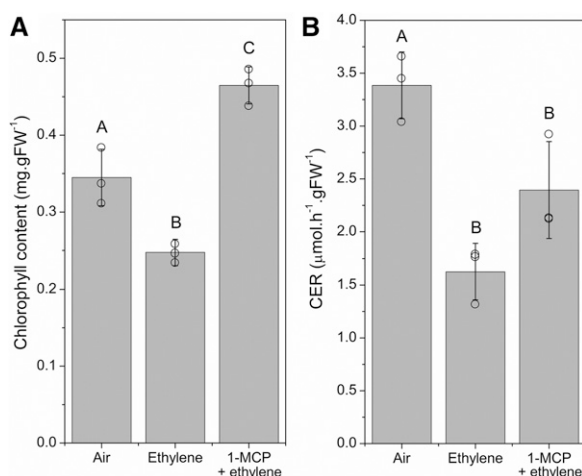


Figure 7. Ethylene reduces chlorophyll content (mg·gFW⁻¹; A) and net photosynthetic capacity (CO₂ exchange rate [CER]; μmol·h⁻¹·gFW⁻¹; B) after 10 d of treatment with 10 ppm ethylene gas. This ethylene effect was reduced by a pretreatment of 10 ppm 1-MCP. Bars represent the average of three biological replicates with SD of the mean. The values of individual replicates are superimposed (white circles) on the bars. Significant differences ($P < 0.05$) are indicated by letters above the bars.

(both up- and down-regulated). This suggested that there might be a link between abiotic stress responses and ethylene responses. We investigated whether the ethylene response is induced in *Spirogyra* when different abiotic stresses are applied: low pH (pH 4.4), osmolarity / nutrient stress (deionized water instead of WC medium), constant light (24-h light, no dark period), salinity (50 mM NaCl), high temperature stress (25°C), and low light stress (10 μmol·m⁻²·s⁻¹). After 10 d the average cell length was measured with and without 1-MCP (10 ppm) treatment. With the exception of high temperature and low light stress, we found that these abiotic stress conditions induced cell elongation, and 1-MCP treatment prevented this elongation response (Fig. 8), indicating that ethylene signaling is essential to achieve the elongation response. We were, however, unable to detect an increase in ethylene production of *Spirogyra* cells as a result of the applied abiotic stress conditions.

Finally, to explore the relationship between abiotic stress, ethylene treatment, and gene expression, we used qPCR to test whether the expression of select genes that are strongly up-regulated at 24 h of ethylene treatment in our RNA-seq data is also up-regulated by abiotic stress at the same time point. We performed qPCR analysis of three cell wall-related transcripts (UDP-D-GLC/UDP-D-GAL-4-EPIMERASE1 [UGE1]), EXPA18 and XTH1, the ethylene-regulated ERF (CRF1), and two abiotic stress responsive transcripts (HEAT SHOCK PROTEIN91 [HSP91] and CHAPERONE DNA-DOMAIN SUPERFAMILY PROTEIN [CDJP]) of *Spirogyra* after 24 h of osmolarity/nutrient stress treatment, both with and without 1-MCP (Supplemental Fig. S11). In this experiment, only the cell wall-related transcripts were significantly up-regulated by the stress treatment

at 24 h. This up-regulation, however, was independent of the 1-MCP treatment (i.e. independent of ethylene signaling). Expression of CRF1, HSP91, and CDJP was unaltered by any of the treatments. These results suggest that ethylene is not involved in the regulation of these particular cell wall-related transcripts at 24 h of H₂O stress and that ethylene-induced expression of CRF1, HSP91, and CDJP might be unrelated to H₂O stress at this time point.

DISCUSSION

The biology of ethylene is well documented for flowering plants, but less is known about the role of ethylene in basal embryophytes and algae. It was recently shown that ethylene signaling has an ancient origin in a common ancestor of *Spirogyra* and land plants (Ju et al., 2015). Here, we investigated the biology of ethylene and the underlying molecular mechanism of the ethylene-induced cell elongation response in *Spirogyra*. In angiosperms, ethylene is well known for its inhibition of cell elongation, for example, in the triple response in dark-grown seedlings (Guzmán and Ecker, 1990). Nonetheless, ethylene can also stimulate cell elongation, depending on tissue- and cell-type specificity and developmental stage. Ethylene-stimulated cell elongation has been reported in a variety of land plants, including mosses, a liverwort, ferns, and angiosperms (Ku et al., 1970; Suge, 1971; Stange and Osborne, 1988; Musgrave and Walters, 1974; Cookson and Osborne, 1979; Métraux and Kende, 1983; Suge et al., 1997; Smalle et al., 1997; Van Der Straeten et al., 2001; Yasumura et al., 2012). The conservation of the elongation response among embryophyte lineages and *Spirogyra* (Ju et al., 2015; and this study) suggests that cell elongation is one of the basic and perhaps original responses to the hormone ethylene. Yet the physiological advantage of cellular elongation in *Spirogyra* remains unknown. Based on our findings, we hypothesize that the ethylene-regulated elongation is a physiological response toward abiotic stress or a changing environment that originated during the early divergence of charophyte algae more than 450 million years ago. Because *Spirogyra* species (and other charophytes) can live in both submerged and subaerial conditions, we propose that the ethylene-regulated elongation response allows portions of the filaments to reach (more) favorable environmental conditions. Perhaps the ability of ancestral charophyte algae to utilize ethylene as a hormone gave them the ability to respond to a changing environment and thus was conceivably a key feature that enabled the successful colonization of land by plants.

Spirogyra Has Relatively Few Ethylene-Responsive Transcription Factors

Ethylene-responsive transcription factors play an important role in transducing the ethylene signal into downstream biological responses. In Arabidopsis, the

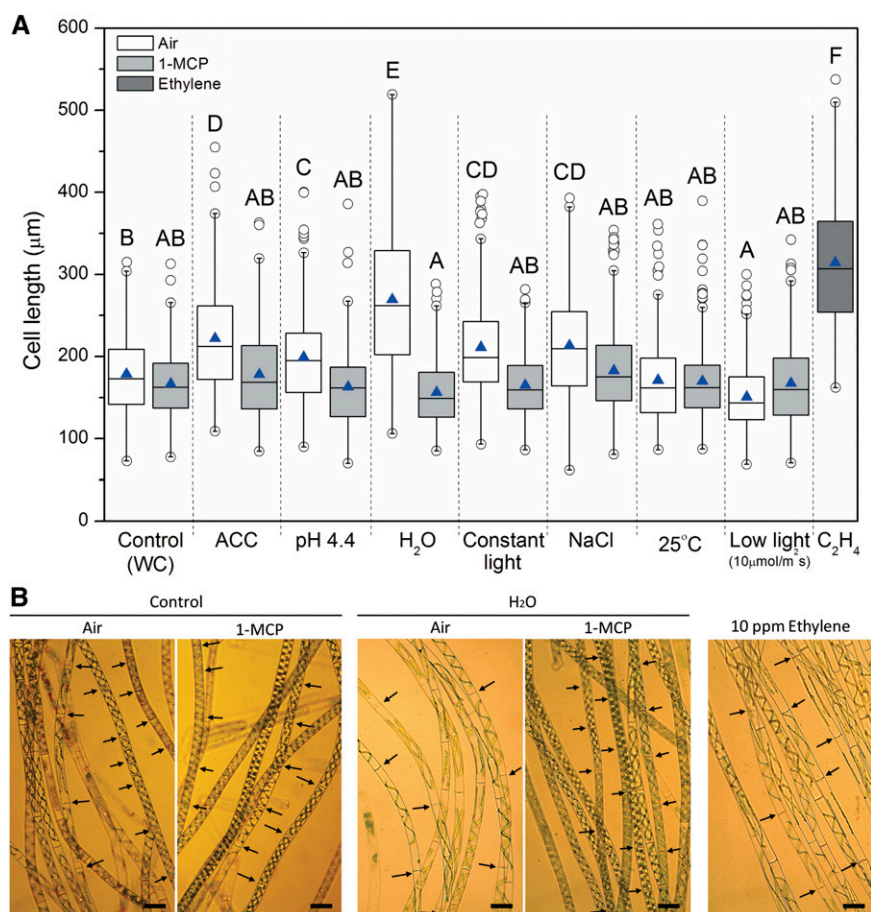


Figure 8. Effect of abiotic stress on cell length of *Spirogyra* after 10 d. A, Different stress treatments (WC medium at low pH 4.4), osmolarity/nutrient (deionized H₂O), constant light (24 h), and salinity (WC medium with 50 mM NaCl) significantly increased the average cell length compared to the no-stress control (WC medium). This cell elongation response was completely inhibited when treated with 1-MCP (10 ppm; gray boxes). Heat stress (25°C) and low light conditions (10 μmol·m⁻²·s⁻¹) do not significantly increase cell length. The two positive controls, ACC (500 μM in WC medium) and ethylene (10 ppm) treatment, also induced the cell elongation response. All boxes bound the 25th and 75th percentiles, whiskers show the outermost data points <1.5 times the interquartile range beyond the box. Blue diamonds indicate the mean; horizontal lines indicate the median; white circles indicate outliers. Significant differences ($P < 0.05$) are indicated by letters above the bars. B, Microscopic images of filaments after 10 d of stress with deionized H₂O compared to the no-stress control (WC medium). The 1-MCP treatment (10 ppm) can inhibit the stress-induced elongation response. Cell lengths were quantified in A. Arrows indicate the ends of individual cells in the filaments. The scale bar is 50 μm.

transcription factors EIN3 and the EILs, together with 147 ERFs, are important signal transducers that activate a myriad of physiological processes upon ethylene signaling (Nakano et al., 2006). The *Spirogyra* transcriptome harbors only 10 transcripts that contain the conserved AP2/ERF domain, suggesting that *Spirogyra* has a smaller number of ERFs. However, because genes that are not, or only weakly, expressed will not be present in the assembled transcriptome, we cannot rule out the existence of additional ERFs in the *Spirogyra* genome. Most of these 10 ERFs did not show an ethylene-responsive expression profile. The transcript annotated as *ERF71* did show a partial up-regulation by ethylene (Supplemental Fig. S6), and it was previously shown by qPCR that this gene could be induced by ethylene treatment in *Spirogyra* (Ju et al., 2015). The only ERF homolog that shows a strong and steady increase in expression upon ethylene treatment, similar to *AtERF1* or *AtERF5* in Arabidopsis (Chang et al., 2013), is the ERF that is annotated as *CRF1* (Supplemental Fig. S6F). In Arabidopsis, CRFs are characterized by a specific cytokinin response domain (CRF domain; Rashotte and Goertzen, 2010), which is absent in the *Spirogyra* CRF1 homolog and even totally absent from the *Spirogyra* transcriptome. Nonetheless, it is clear that this *CRF1* homolog is the only ERF homolog that is very strongly up-regulated by ethylene in *Spirogyra*. The actual number and expression

patterns of ERFs might be linked with the increase in diversification of function and species complexity (multicellularity, terrestrial habitat) that occurred during plant evolution (Chen and Rajewsky, 2007).

Ethylene Regulates *Spirogyra* Cell Wall Modification

The link between cell elongation and ethylene in algae has been largely unexplored, despite extensive research on cell wall composition and evolution in algae (Sørensen et al., 2010; Mikkelsen et al., 2014). Cellular elongation requires relaxation of the cell wall in order to facilitate turgor-driven longitudinal expansion (Cosgrove, 2000). Our mRNA-seq data uncovered a group of cell wall remodeling enzymes that appear to be differentially regulated by ethylene at the gene expression level in *Spirogyra*. This mainly includes expansins and xyloglucan endotransglucosylases/hydrolases. Both of these enzymes are involved in the relaxation of the cell wall matrix facilitating cellular elongation (Li et al., 2003; Bashline et al., 2014). The target polymers of EXPs (cellulose) and XTHs (xyloglucan) were previously shown to be present in cell walls of charophytes including *Spirogyra* (Sørensen et al., 2011). We also showed that 24 h of osmolarity/nutrient stress (H₂O treatment) slightly induced the expression of two homologs (*XTH1* and *EXPA18*) of these multigene

families, as well as another cell wall modifying gene (*UGE1*). *EXPs* and *XTHs* are ethylene regulated in angiosperms such as *Arabidopsis* (Cho and Cosgrove, 2002; Polko et al., 2012; Rauf et al., 2013; Vissenberg et al., 2001), rice (Cho and Kende, 1997), and maize (Saab and Sachs, 1996; Kam et al., 2005). *EXPs* and *XTHs* are also ethylene regulated during climacteric fruit ripening, resulting in fruit softening (Rose and Bennett, 1999). The fact that *Spirogyra* also harbors ethylene-regulated *EXPs* and *XTHs* might indicate that these genes were among the first ethylene-regulated genes in the common ancestor of land plants and charophyte algae.

Ethylene Down-Regulates the Photosynthetic Capacity of *Spirogyra*

Besides elongation and abiotic stress, our data also support the hypothesis that ethylene down-regulates chlorophyll biosynthesis and photosynthesis in *Spirogyra*. The effect of ethylene on photosynthesis in embryophytes appears to be highly species specific, with some species showing a severe down-regulation of the photosynthetic capacity, while other are less affected or insensitive (Pallas and Kays, 1982; Taylor and Gunderson, 1986). Early work in *Arabidopsis* showed that ethylene enhances leaf senescence (Bleecker et al., 1988) and that the ethylene-insensitive mutants *etr1* and *ein2* have a slower senescence rate compared to wild-type plants (Guzmán and Ecker, 1990; Grbid and Bleecker, 1995). This is likely caused by rapid chlorophyll breakdown as observed in ethylene-treated *Arabidopsis* plants (Zacarias and Reid, 1990). Ethylene also down-regulates photosynthetic genes in *Arabidopsis* (Grbid and Bleecker, 1995). On the other hand, ethylene-insensitive mutants seem to have a lower whole-plant photosynthesis rate (Tholen et al., 2004). Together with our observations in *Spirogyra*, it is plausible that the ethylene-mediated regulation of photosynthesis is an ancient link that originated prior to the colonization of the land. Yet the biological significance of down-regulating plant photosynthesis by ethylene remains speculative.

Abiotic Stress Responses Crosstalk with Ethylene in *Spirogyra*

In embryophytes, rapid ethylene-regulated cell elongation is often a growth response to a changing environment, such as shade (Pierik et al., 2007) or flooding (Voesenek and Bailey-Serres, 2015). Our study has revealed that certain abiotic stress conditions (salinity, pH, light, and osmolarity/nutrient stress) can induce cellular elongation through the action of ethylene signaling. We also showed that ethylene regulates numerous stress-responsive genes in *Spirogyra*, especially related to abiotic stress. We hypothesize that during abiotic stress, cellular elongation could serve as a useful physiological trait that allows the alga to grow and reach more favorable conditions, such as light or oxygen. Studies on the moss *P. patens* have shown that

a submergence-induced “escape response” (consisting of a shift during protonemal growth from central, highly photosynthetic chloronema toward spreading, less photosynthetically active caulonema, and more peripheral induction of gametophores, which are the leafy shoots of mosses) is regulated by ethylene (Yasumura et al., 2012). In the charophyte alga *Klebsormidium crenulatum*, desiccation stress regulates many desiccation tolerance genes also known in land plants (Holzinger et al., 2014), and the genome of *K. flaccidum* indicates the presence of many stress-related genes that could be used to sense and respond to environmental changes (Hori et al., 2014). Despite this potentially conserved link between ethylene and abiotic stress responses, our qPCR analysis indicated that the expression of some cell wall-related homologs (*UGE1*, *EXPA18*, and *XTH1*), a *CRF1* homolog, and two abiotic stress homologs (*HSP91* and *CDJP*) were not ethylene regulated at 24 h of osmolarity/nutrient stress (by means of an H₂O treatment). It is possible that the timeframe of 24 h was not suitable to capture the effect of stress-induced ethylene on gene expression, or, alternatively, other genes are ethylene-regulated during stress conditions. It remains unknown exactly how stress and ethylene signaling are intertwined in *Spirogyra*.

We also did not detect an increase in the production of ethylene in stress-treated *Spirogyra*, although abiotic stress typically induces ethylene production in embryophytes (Kazan, 2015). A similar observation was made for the aquatic monocot *Potamogeton pectinatus* (fennel pondweed), which failed to produce detectable levels of ethylene when given different stress or hormone treatments (Summers et al., 1996) and yet responds to submergence by stem elongation like many aquatic and semiaquatic species (Summers and Jackson, 1994). There are several possible explanations for why abiotic stress did not result in a detectable increase in ethylene production in *Spirogyra*. Most likely, the amount of ethylene produced was very low and/or ethylene was completely solubilized in the cells. Because the diffusion coefficient of ethylene in water is about 10,000 times lower than in air (Jackson, 1985), ethylene diffusion outside the cells is hampered in water, leading to a rapid accumulation of intracellular ethylene. Due to the high affinity of ethylene for phospholipids (membranes), cellular ethylene likely disperses throughout the filament via the endomembrane system and plasmodesmata. Ethylene would be expected to accumulate in submerged filaments but would rapidly decrease in intracellular concentration when any portion of the filament is in contact with the atmosphere; under this model, ethylene would provide a sensitive system to detect emergence. Alternatively, abiotic stress responses might be mediated by another hormone, such as abscisic acid, which crosstalks with ethylene signaling. Interestingly, it has recently been shown that the CONSTITUTIVE TRIPLE RESPONSE1 (*CTR1*) homolog in *P. patens* has a dual signaling role for both ethylene and abscisic acid, but this is not the case for angiosperms (Yasumura et al., 2015). Another hypothesis could be that *Spirogyra*

produces a different small hydrocarbon signaling molecule, such as propylene or methyl isocyanide (Burg and Burg, 1967; Sisler and Yang, 1984), which is able to activate the ethylene receptor.

CONCLUSION

Our work has shed light on the function of ethylene as a hormone in the charophyte alga *S. pratensis* and its role in regulating the expression of different pathways. There are many similarities between the ethylene responses in *Spirogyra* and land plants despite their evolutionary distance. We have shown that ethylene regulates shared processes, including cell wall remodeling, chlorophyll biosynthesis, photosynthesis, and abiotic stress responses. The conserved role of ethylene as a hormone in *Spirogyra* and land plants suggests that the ancient ancestors of both land plants and *Spirogyra* were among the first plants to have obtained this ethylene regulatory mechanism. One can even postulate that the acquisition of a hormonal system, such as ethylene, by charophyte algae was crucial for the colonization of land by plants. Although *Spirogyra* has a large set of ethylene-regulated genes (828 differentially expressed transcripts after 24 h of ethylene treatment), we identified only 10 different *ERF/AP2* transcription factor homologs, of which only one appears to be strongly and steadily up-regulated by ethylene. This finding suggests that ethylene responses in *Spirogyra* are regulated by only a small number of ERFs and that the vast diversity of ERFs observed in, for example, *Arabidopsis* arose as green algae evolved into multicellular species and acquired new habitats. By studying hormonal systems in charophyte algae such as *Spirogyra*, one can uncover novel insights in basic hormonal responses, which can help to advance our understanding of the evolution of plants.

MATERIALS AND METHODS

Plant Material and Experimental Design

Liquid cultures of *Spirogyra pratensis* Transseau (UTEX 928) were grown in Guillard's Freshwater medium (WC; Andersen et al., 2005), pH 7.9, in a culture chamber at 18°C under white light with a photoperiod of 14-h-light (7:00 AM to 9:00 PM) and 10-h-dark (9:00 PM to 7:00 AM) cycle. Artificial light was supplied with a photon flux of 180 to 200 $\mu\text{mol} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$. All treatments were applied to "semi-dry" cultures of *Spirogyra*, which were made by transferring filaments from liquid culture to petri dishes or glass vials and kept moist by adding 400 μL of WC medium (Supplemental Fig. S1). This semidry method was intended to allow for a more efficient and rapid diffusion of ethylene gas into the medium, facilitating exposure of the filaments to ethylene.

A total of 24 samples were used for the RNA sequencing experiment. Samples were made from filaments taken from liquid cultures of the same age, which were transferred to petri dishes in semidry conditions. One-half of the petri dishes were exposed to 10 ppm ethylene by injecting ethylene gas in 1-L airtight transparent jars, and the other one-half were exposed to ambient air (control) in identical jars. The samples were treated at 8:00 AM (1 h after starting the light period). Sampling of both the control (ambient air) and treated (ethylene) samples was carried out for three independent samples at 3 h (11:00 AM), 6 h (2:00 PM), 12 h (8:00 PM; 1 h before start dark period), and 24 h (8:00 AM the next day) after treatment. All samples were taken during the photoperiod.

RNA Extraction, mRNA-seq Library Preparation, Sequencing, and Data Processing

Total RNA was extracted from *Spirogyra* filaments using Nucleospin RNA Plant isolation kit (Macherey-Nagel) following the manufacturer's protocol. RNA quality was assessed using the Experion Electrophoresis System (Bio-Rad) with Experion RNA StdSens chips (Bio-Rad). RNA quantity was measured with a Nanodrop spectrophotometer (Thermo Scientific). Reverse transcription was carried out with the iScript cDNA Synthesis Kit (Bio-Rad) following the manufacturer's protocol. Purification of mRNA was carried out with the Poly(A) mRNA Magnetic Isolation Module (New England Biolabs) using Agencourt AMPure XP magnetic beads (Beckman Coulter). DNA fragment libraries were prepared for Illumina HiSeq using the NEB Next Ultra RNA Library Prep Kit for Illumina (New England Biolabs) following the manufacturer's protocol. To multiplex the 24 samples on two lanes of the Illumina HiSeq, we used the Next Multiplex Oligos for Illumina (New England Biolabs) for primer indexing. The adaptor sequence, universal primer sequence, and indexing primer sequences are given in Supplemental Table S1. Sequencing was carried out by the University of Maryland Institute for Bioscience and Biotechnology Research Sequencing Core facility on a HiSeq1500 (Illumina) sequencer to generate 101 base, paired-end reads.

Further information about the initial processing of the raw RNA-seq reads, sequence annotation, differential gene expression analysis, and qPCR validation is described in the Supplemental Methods.

Chlorophyll Content and Photosynthesis

Spirogyra filaments originating from a different batch were transferred from liquid culture to semi-dry conditions in petri dishes and kept moist with 400 μL WC medium. The petri dishes were enclosed in airtight jars and treated by injecting ethylene (10 ppm final concentration) or 1-MCP (10 ppm final concentration; gift from Mark Tucker, USDA-ARS), a potent inhibitor of ethylene perception, or left untreated (control). Both chlorophyll content and photosynthesis were measured on three biological replicates per treatment.

Total chlorophyll content was extracted from 50 mg fresh tissue (ground in liquid nitrogen) with 1 mL of 80% ethanol and 5 mM HEPES for 10 min at 50°C. Cell debris was removed by centrifugation for 5 min at 21,000 g . Absorption was measured for chlorophyll *a* at 664 nm and chlorophyll *b* at 649 nm using a spectrophotometer (Bio-Rad). Total chlorophyll content was calculated using the formula given by Lichtenthaler and Buschmann (2001).

Net photosynthetic capacity was measured with the flow-through Q-box CO650 (Qubit Systems). *Spirogyra* tissue was placed on a wetted nitrocellulose membrane in the flow-through chamber. The chamber was continuously flushed with 350 ppm CO_2 containing air at a flow rate of 0.05 $\text{L} \cdot \text{min}^{-1}$, and illuminated with the A113 led light at 1428 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. After 6 min the CO_2 output was stabilized, and the CO_2 exchange rate was calculated with the Logger Pro software (Qubit Systems).

Ethylene Production

Spirogyra was transferred from standard liquid culture into an airtight 10-mL vial (Supplemental Fig. S1) and kept moist with 400 μL WC medium. After 48 h, a gas sample (1 mL) from the vial headspace was injected into a HP 6890 gas chromatograph (Agilent) equipped with a Flame Ionization Detector to detect ethylene. The retention time for ethylene was calibrated with reference ethylene gas (10 and 1 ppm; Praxair). Alternatively, ethylene production was assessed with photo-acoustic laser spectroscopy using the ETD-300 (SensorSense) in similar 10-mL vials. Ethylene content in the headspace was quantified after 6 or 24 h of incubation.

Stress Treatments

Spirogyra filaments were transferred from liquid WC cultures to semidry conditions in petri dishes and kept moist with 400 μL liquid WC medium. For each condition, we carried out three biological replicates. For the negative control treatment, the filaments were kept moist with 400 μL of WC media. For the different stress conditions, the filaments were treated with (1) 400 μL of a salt solution (WC with 50 mM NaCl), (2) a low-pH solution (WC pH 4.4), or (3) deionized water (osmolarity and nutrient stress). Other stress conditions were applied by high temperature (25°C), constant light (no day/night cycles), and low light (10 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$). A positive control was obtained by treating the filaments with the ethylene precursor ACC (WC with 500 μM ACC) or with 10 ppm ethylene. To verify the role of ethylene during stress conditions, one-half of the samples were treated with 10 ppm 1-MCP. After 10 d of stress

conditions, digital images of the filaments were taken using a Canon 5D Mark II digital camera attached to a Zeiss Axioskop microscope with a 2.5× phototube and a 20× objective.

Cell Length Measurements

Cell length was measured using ImageJ (<http://rsbweb.nih.gov/ij/>) on digital images taken under a microscope (Zeiss Axioskop, see above). Between 200 and 300 individual cells were measured for each stress condition, depending on the number of cells visible in each image.

Statistical Analyses

For statistical analyses, we used Statistical Software Analysis (SAS Enterprise guide 6.1) with the ANOVA and linear models procedures using the Tukey means comparison test with a confidence interval of $\alpha = 0.05$ and all parameters set to default.

Accession Numbers

The 24 *S. pratensis* transcriptome sequencing files are deposited in the NCBI Sequence Read Archive under the SRA study number SRP081241.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Overview of the experimental setups.

Supplemental Figure S2. Differential expression analysis of all contigs.

Supplemental Figure S3. Correlation matrix of the different treatments.

Supplemental Figure S4. Volcano plots of untreated samples.

Supplemental Figure S5. Validation of expression profiles by qPCR.

Supplemental Figure S6. Expression profiles of the AP2/ERF domain containing homologs.

Supplemental Figure S7. Sequence alignments the *Spirogyra* and Arabidopsis ERFs.

Supplemental Figure S8. GO enrichment analysis of the 12- and 6-h time point.

Supplemental Figure S9. MapMan analysis of the 24-h time point.

Supplemental Figure S10. Expression profiles chlorophyll biosynthesis and photosynthesis related homologs.

Supplemental Figure S11. Gene expression during water stress treatment.

Supplemental Table S1. List of primers used in the study.

Supplemental Table S2. Summary of the raw mRNA sequencing data.

Supplemental Table S3. Summary of the *S. pratensis* assembly statistics.

Supplemental Table S4. List of qPCR primers.

Supplemental File S1. List of differentially expressed genes for all time points.

Supplemental File S2. TMM-FPKM expression profiles of all *Spirogyra* transcripts.

Supplemental Materials and Methods. Information on the initial processing of raw RNA-seq reads, sequence annotation, differential gene expression analysis, and the supplemental references used.

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